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Signal Transducer and Activator of Transcription 4 Is Required for the Transcription Factor T-bet to Promote T Helper 1 Cell-Fate Determination

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SUMMARY

Transcriptional regulatory networks direct the development of specialized cell types. The transcription factors signal tranducer and activator of transcription 4 (Stat4) and T-bet are required for the interleukin-12 (IL-12)-stimulated development of T helper 1 (Th1) cells, although the hierarchy of activity by these factors has not been clearly defined. In this report, we show that these factors did not function in a linear pathway and that each factor played a unique role in programming chromatin architecture for Th1 gene expression, with subsets of genes depending on Stat4, T-bet, or both for expression in Th1 cells. T-bet was not able to transactivate expression of Stat4-dependent genes in the absence of endogenous Stat4 expression. Thus, T-bet requires Stat4 to achieve complete IL-12-dependent Th1 cell-fate determination. These data provide a basis for understanding how transiently activated and lineage-specific transcription factors cooperate in promoting cellular differentiation.

INTRODUCTION

The proper development and function of T helper cells is a central requirement for the generation of appropriate immune responses to pathogens and foreign molecules. The differentiation of T helper cells to effector subsets is directed by transcription factors that are capable of programming the expression of genes that are required for specialized functions of a subset of cells (Murphy and Reiner, 2002; Ansel et al., 2003). T helper 1 (Th1) cell differentiation is promoted by stimulation with interelukin-12 (IL-12) and the subsequent activation of signal transducer and activator of transcription 4 (Stat4) (Hsieh et al., 1993; Kaplan et al., 1996; Thierfelder et al., 1996). The T-box transcription factor T-bet has been termed a "master regulator" of Th1 cell development (Szabo et al., 2000; Szabo et al., 2002), and expression is induced during Th1 cell differentiation by interferon γ (IFNγ)-stimulated Stat1 activation (Lighvani et al., 2001; Afkarian et al., 2002). The susceptibility of Stat4- and T-bet-deficient mice to intracellular pathogens and the resistance of these mice to the development of inflammatory disease support a model wherein Stat4 and T-bet are required for the normal development and/or function of Th1 cells (Szabo et al., 2002; Kaplan, 2005; Sullivan et al., 2005).

The development of specialized cells requires networks of transcription factors that work together to mediate changes in gene expression to determine cell fate (Laiosa et al., 2006; Rothenberg, 2007). Although Stat4 and T-bet are required for development of Th1 cells, the coordination of Th1 gene programming by these factors has not been well studied. In the absence of Stat4 or T-bet, there is decreased histone acetylation and increased DNA methylation of Th1 genes, including *Ifng* and *Il18r1* (Avni et al., 2002; Fields et al., 2002; Chang and Aune, 2005; Yu et al., 2007), and ectopic T-bet expression can induce histone modification and chromatin remodeling, even in the absence of Stat4 (Mullen et al., 2001; Shnyreva et al., 2004; Tong et al., 2005). It is not clear, however, whether Stat4 and T-bet operate in linear or parallel pathways to the Th1 cell phenotype. In a linear-pathway model represented by IL-12-Stat4-IFNγ-Stat1-T-bet-IFNγ, Stat4 provides a transient increase in IFNγ that is then able to induce T-bet expression, which in turn potentiates *Ifng* expression (Usui et al., 2003). In a linear pathway, it is also possible that Stat4 has transient effects on chromatin that allow access to other factors that mediate sustained gene programming. Indeed, Stat4 mediates transient histone acetylation of the *Il2ra, IL12RB2*, and *Il18r1* genes (O'Sullivan et al., 2004; Letimier et al., 2007; Yu et al., 2007). A separate though not mutually exclusive pathway suggests that T cell receptor (TCR) and IFNγ signaling promote T-bet expression and induce *Il12rb2* expression to facilitate IL-12 and Stat4 function (Mullen et al., 2001). However, several reports suggest that these models do not completely define the relative roles of Stat4 and T-bet in Th1 cell differentiation. First, it is not clear that T-bet is required for *Il12rb2* expression (Usui et al., 2006). Moreover, although overexpression of T-bet in Stat4-deficient T cells can induce IFNγ expression and histone acetylation, it does not recapitulate wild-type IFNγ expression or *Ifng* acetylation by itself (Mullen et al., 2001; Fields et al., 2002). Despite the proposal that Stat4 mainly plays a role in Th1 cell expansion or survival downstream

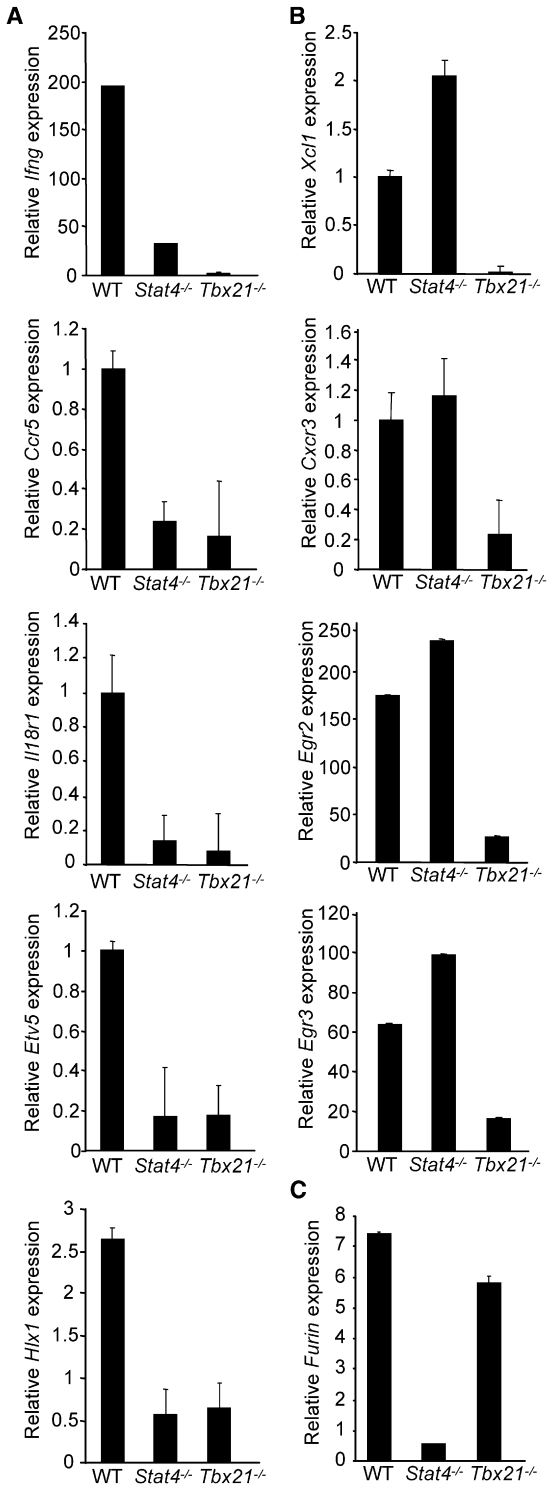


Figure 1. Contribution of Stat4 and T-bet to Expression of Genes in Th1 Cells

Wild-type, Stat4-deficient (*Stat4*$^{-/-}$), and T-bet-deficient (*Tbx21*$^{-/-}$) CD4$^+$ T cells were cultured under Th1 cell conditions (IL-12 + anti-IL-4) for 5 days. RNA was isolated from cells either before (*Ccr5*, *Il18r1*, *Etv5*, and *Cxcr3*) or 6 hours after (*Ifng*, *Hlx1*, *Xcl1*, *Egr2*, *Egr3*, and *Furin*) restimulation of cells with anti-CD3. Quantitative PCR with TaqMan primers specific for each gene was performed, and results were normalized to expression of beta2-microglobulin. Genes are grouped according to dependence on Stat4 and T-bet (A), T-bet only (B), or Stat4 only (C). Results are the average ± SD of replicate samples and are representative of four experiments with similar results.

of T-bet (Mullen et al., 2001; Murphy and Reiner, 2002; Ansel et al., 2003), Stat4 is activated in T-bet-deficient cells, and transduction of Stat4 into differentiating T-bet-deficient T cells results in increased IFNγ production, suggesting that Stat4 has some effects even in the absence of T-bet (Usui et al., 2006; Zhang and Boothby, 2006). Thus, the functional relationship between Stat4 and T-bet in developing Th1 cells may be more complex than is currently appreciated.

In this report, we examine the relative roles of Stat4 and T-bet in Th1 gene programming. In examining many genes associated with the Th1 program, we find subsets that require both Stat4 and T-bet or only one of the factors. Stat4-dependent gene expression could not be rescued by supplemental IFNγ, and T-bet was capable of binding some common target genes in the absence of Stat4. Chromatin modifications to Th1 genes were altered in the absence of either factor, although specific modifications were affected more by one factor than the other. Moreover, ectopic T-bet expression was able to rescue Th1 gene expression and histone acetylation in T-bet-deficient T cells, but not in Stat4-T-bet-double-deficient T cells, supporting a model wherein both Stat4 and T-bet are required for complete activation of the Th1 cell phenotype.

RESULTS

Stat4 and T-bet Regulation of Th1 Gene Expression

To define the relative roles of Stat4 and T-bet in the differentiation of Th1 cells, we systematically analyzed the mRNA expression of genes previously described as having Th1 cell-restricted expression (Table S1 available online). CD4$^+$ T cells from C57BL/6, *Stat4*$^{-/-}$, or *Tbx21*$^{-/-}$ mice were differentiated under Th1 cell culture conditions for 5 days, and gene expression was analyzed with quantitative real-time polymerase chain reaction (RT-PCR) either in resting Th1 cells or in Th1 cells activated for 6 hours with anti-CD3, the latter when expression was low or undetectable in resting cells. We found that the expression of *Ifng*, *Ccr5*, *Il18r1*, *Hlx1*, and *Etv5* were largely dependent upon the presence of both Stat4 and T-bet (Figure 1A). In contrast, *Xcl1, Cxcr3, Egr2*, and *Egr3* were decreased in *Tbx21*$^{-/-}$ cultures but had normal expression in the absence of Stat4 (Figure 1B). *Furin*, which has previously been shown to be Stat4 dependent (Pesu et al., 2006), is independent of T-bet (Figure 1C). Similar results were observed in cultures of naive CD4$^+$ T cells (Figure S1A). These results demonstrate that Stat4 and T-bet regulate both overlapping and distinct subsets of Th1 genes.

Whether T-bet expression is actually decreased in Stat4-deficient Th1 cell cultures has been somewhat controversial (Mullen et al., 2001; White et al., 2001; Afkarian et al., 2002; Hoey et al., 2003), and it is possible that the decreased endogenous IFNγ in Stat4-deficient Th1 cell cultures contributes to decreased Th1 cell development in the absence of Stat4 (Usui et al., 2003). To directly test whether decreased IFNγ is responsible for the phenotype of *Stat4*$^{-/-}$ cultures, we incubated wild-type or *Stat4*$^{-/-}$ T cells under Th1 cell conditions in the presence or absence of supplemental IFNγ. After 5 days of culture, cells were washed

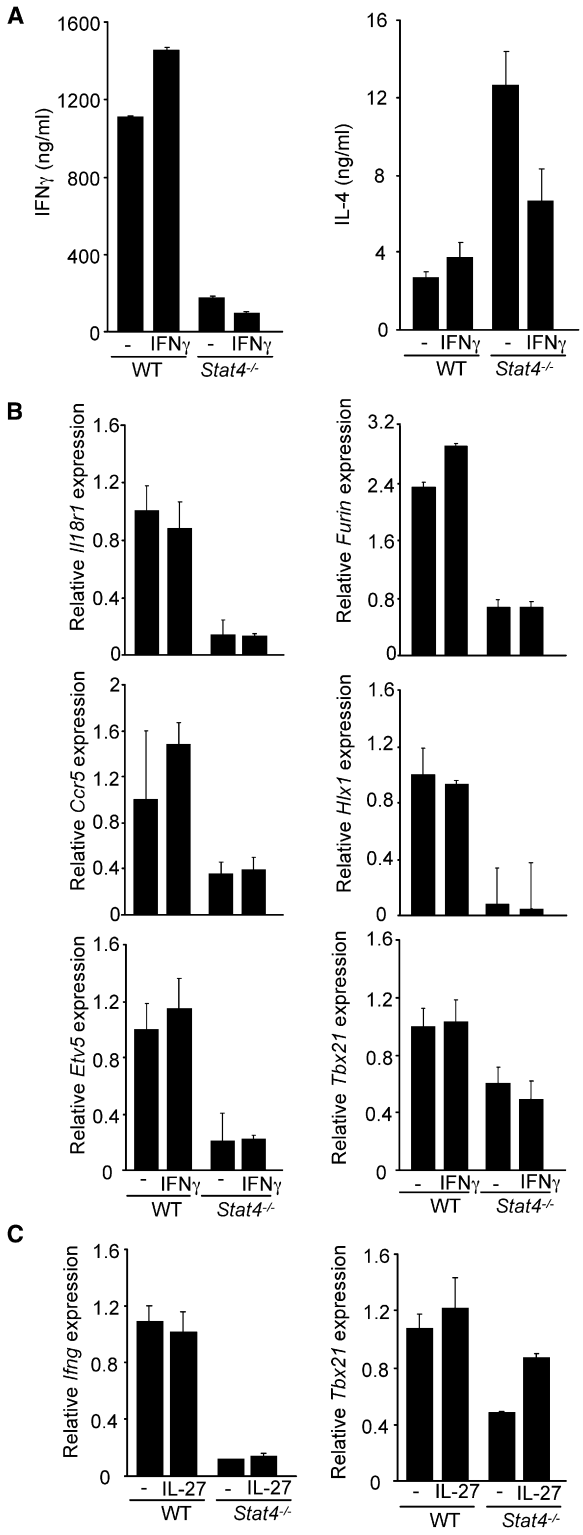


Figure 2. IFNγ or IL-27 Do Not Rescue Gene Expression in Stat4-Deficient Th1 Cells

(A) Wild-type and Stat4-deficient (*Stat4*$^{-/-}$) CD4$^+$ T cells were cultured under Th1 cell conditions (IL-12 + anti-IL-4) in the presence or absence of 100 ng/ml recombinant IFNγ for 5 days. Cells were restimulated with anti-CD3 for 18 hr, and supernatants were analyzed for IFNγ and IL-4 with ELISA.

(B) Cells cultured as in (A) were analyzed for gene expression with quantitative PCR as described in Figure 1. Results in (A) and (B) are the average ± SD of replicate samples and are representative of four experiments with similar results.

(C) Wild-type and Stat4-deficient (*Stat4*$^{-/-}$) CD4$^+$ T cells were cultured under Th1 cell conditions (IL-12 + anti-IL-4) in the presence or absence of 100 ng/ml recombinant IL-27 for 5 days. Expression of genes was determined after activation with anti-CD3 for 4 hr. Results are representative of two experiments with similar results.

and restimulated before IFNγ production was assessed by enzyme-linked immunosorbent assay (ELISA) and gene expression was assessed by quantitative PCR. The addition of IFNγ to Stat4-deficient cultures did not alter the production of IFNγ from restimulated Stat4-deficient Th1 cell cultures (Figure 2A). Adding IFNγ activated Stat1 and decreased the amount of IL-4 produced from Stat4-deficient cultures (Figure 2A and data not shown), agreeing with previous reports on increased IL-4 production in *Stat4*$^{-/-}$ Th1 cell cultures and the ability of IFNγ to repress IL-4 in Th1 cell cultures (Kaplan et al., 1996; Zhang et al., 2001). This finding also confirms that IFNγ added to these cultures was present in biologically active amounts. Adding exogenous IFNγ to Stat4-deficient T cell cultures did not rescue gene expression of *Il18r1*, *Ccr5*, *Etv5*, *Furin*, or *Hlx1* (Figure 2B). We did observe a modest decrease in T-bet expression in the absence of Stat4, and expression was not recovered by the addition of IFNγ.

Because T-bet expression was modestly decreased in these cultures and not rescued by IFNγ addition to the culture, we wanted to further test whether the addition of IL-27, another cytokine implicated in Th1 cell development that induces T-bet expression in a Stat1-dependent manner (Takeda et al., 2003), to Stat4-deficient cultures could recover any of the phenotype. Despite IL-27-induced *Tbx21*, IL-27 did not increase *Ifng* expression in Stat4-deficient Th1 cell cultures (Figure 2C). Thus, neither a lack of endogenous IFNγ production nor the modest decrease in T-bet expression is the sole defect in Th1 cell generation by *Stat4*$^{-/-}$ T cells.

Stat4 and T-bet Remodel *Hlx1*

These experiments suggest that Stat4 and T-bet function independently in parallel pathways promoting Th1 cell development. To further explore the relative roles of these factors in programming Th1 gene expression, we chose one gene, *Hlx1*, for detailed study. Hlx cooperates with T-bet in IFNγ production, even in the absence of Stat4, and probably plays an important role in Stat4- and T-bet-dependent programming of *Ifng* expression (Mullen et al., 2002; Martins et al., 2005). Expression of *Hlx1* is decreased in Stat4- and T-bet-deficient cultures throughout the period of Th1 cell differentiation (Figure 3A). The peaks of *Hlx1* expression at days 2 and 4 probably represent direct induction by Stat4 correlating to the addition of IL-12 to cultures on the first and third days of culture. Through the same time period, we also noted the lower expression of *Stat4* in T-bet-deficient cultures (Underhill et al., 2005; Usui et al., 2006) and observed that like T-bet expression in the absence of Stat4, decreased expression is most dramatic early during differentiation (Figure 3A).

We next defined the ability of Stat4 and T-bet to bind to the *Hlx1* promoter, as well as the characterized promoters of *Ifng*

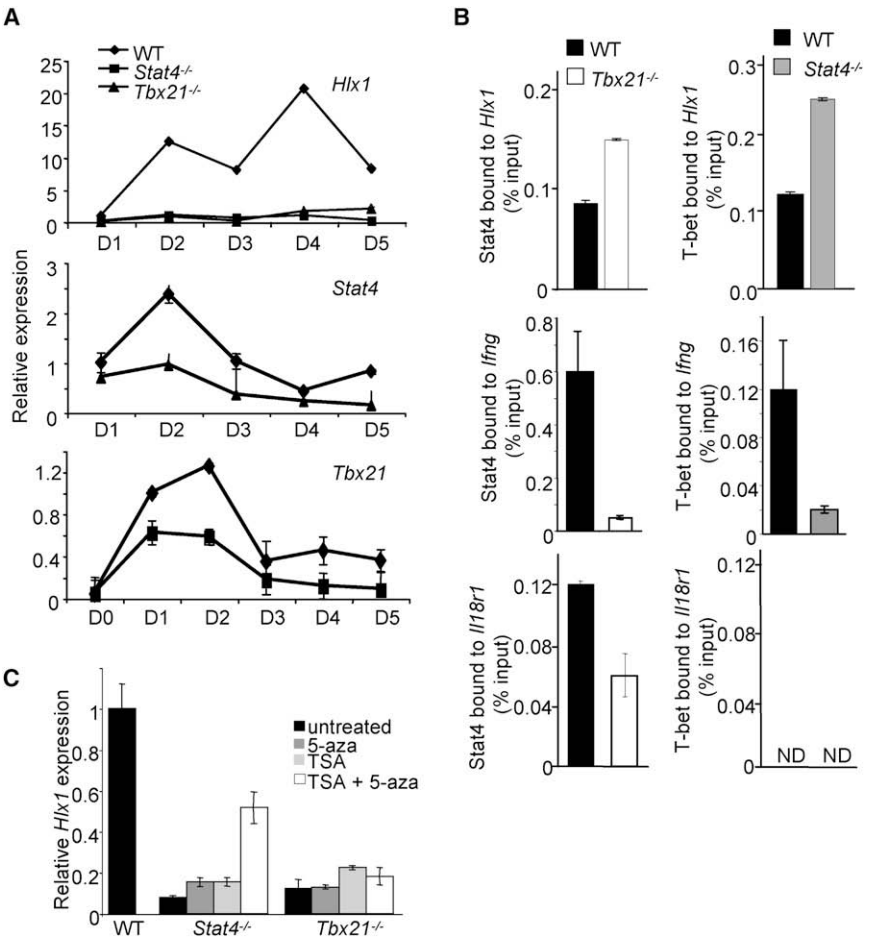


Figure 3. Stat4 and T-bet Bind to the *Hlx1* Locus

(A) Wild-type, Stat4-deficient (*Stat4*$^{-/-}$), and T-bet-deficient (*Tbx21*$^{-/-}$) CD4+ T cells were cultured under Th1 cell conditions (IL-12 + anti-IL-4) for 5 days, and RNA was isolated from cells during each day of culture. Expression of *Hlx1, Stat4*, and *Tbx21* were assessed in each of the samples with quantitative PCR. Results are representative of two experiments.

(B) Wild-type, Stat4-deficient (*Stat4*$^{-/-}$), and T-bet-deficient (*Tbx21*$^{-/-}$) CD4^{+} T cells were cultured under Th1 cell conditions (IL-12 + anti-IL-4) for 5 days, and chromatin was isolated for ChIP assay. ChIP was performed for Stat4 bound to the promoter of *Hlx1*, *Ifng*, or *Il18r1* in wild-type and T-bet-deficient cells (left) or for T-bet bound to the same regions in wild-type or Stat4-deficient cells (right). Quantitative PCR was performed with TaqMan primers specific for each promoter. Transcription factor bound to the locus is expressed as the percentage of the input used for the ChIP assay. Results are the average ± SD of replicate samples and are representative of three experiments for *Hlx1* and two experiments for binding to other promoters with similar results. ND, not detected.

(C) Wild-type, Stat4-deficient (*Stat4*$^{-/-}$), and T-bet-deficient (*Tbx21*$^{-/-}$) CD4^{+} T cells were cultured under Th1 cell conditions (IL-12 + anti-IL-4) for 5 days in the presence or absence of 20 nM trichostatin A (TSA) and/or 10 μM 5-aza-deoxycytidine (5-aza). RNA was isolated for analysis of *Hlx1* gene expression as described in Figure 1. Results are representative of two experiments.

and *Il18r1* in the absence of the reciprocal factor (Chang and Aune, 2007; Schoenborn et al., 2007; Yu et al., 2007). There are a number of conserved noncoding sequences in the promoter and intron 3 of *Hlx1* that we used for primer design (Figure S2). Using ChIP and qPCR to detect binding to the *Hlx1* promoter and intron 3 in Th1 cell cultures at day 3, we detected Stat4 binding at both regions, and binding was increased in the absence of T-bet (Figure 3B and data not shown). Similarly, T-bet binding was detected at both regions, and binding was enhanced in the absence of Stat4 (Figure 3B and data not shown). However, binding patterns of Stat4 and T-bet were distinct at other promoters. There was decreased binding of Stat4 and T-bet to *Ifng*, respectively, in *Tbx21*$^{-/-}$ and *Stat4*$^{-/-}$ Th1 cell cultures compared to binding in control cultures (Figure 3B). Stat4 binding to *Il18r1* was partially affected by T-bet deficiency, and we did not observe binding of T-bet to *Il18r1*, even in wild-type cells (Figure 3B). To further illustrate specificity for Stat4 and T-bet binding, we also tested the association of other STAT and T-box factors to *Hlx1*. Whereas we observed association of Stat1 at less than 50% of Stat4 binding, we did not observe association of Stat6 or Tbx5 at the *Hlx1* promoter (data not shown). These results suggest there are gene-specific effects of Stat4- or T-bet deficiency on the binding of other factors to target loci and that the ability of Stat4 to bind a gene and promote histone modifications is not required to allow accessibility for T-bet at all loci.

Because both factors bind to *Hlx1* in the absence of the reciprocal factor and can mediate epigenetic modifications, we next tested whether altering epigenetic modifications to DNA and histones would recover expression or support a role for either factor in directly promoting transcription. *Stat4*$^{-/-}$ and *Tbx21*$^{-/-}$ T cells were cultured in the absence, presence, or combination of histone deacetylase and DNA methylation inhibitors in parallel with wild-type Th1 cell cultures. Whereas each inhibitor had modest effects on *Hlx1* mRNA in *Stat4*$^{-/-}$ cells, the combination of both inhibitors increased mRNA to approximately half of that observed in wild-type cells (Figure 3C). The inhibitors had less of an effect in the *Tbx21*$^{-/-}$ cells, suggesting that T-bet may be required for regulating transcription as well as remodeling chromatin.

If T-bet and Stat4 were in a linear pathway, we would expect that chromatin modifications mediated by either factor would be similar. Conversely, distinct changes mediated by each factor would support a model wherein they act independently. To define the changes in chromatin that are mediated by Stat4 and T-bet, we examined histone modifications and histone-modifying enzymes at the *Hlx1* locus in wild-type, Stat4-, and T-bet-deficient cells. Total aceytlation of H3 and H4 was decreased at the *Hlx1* promoter in the absence of either Stat4 or T-bet on days 3–5 of differentiation (Figures 4A and 4B). To determine whether the decrease in histone acetylation was due to

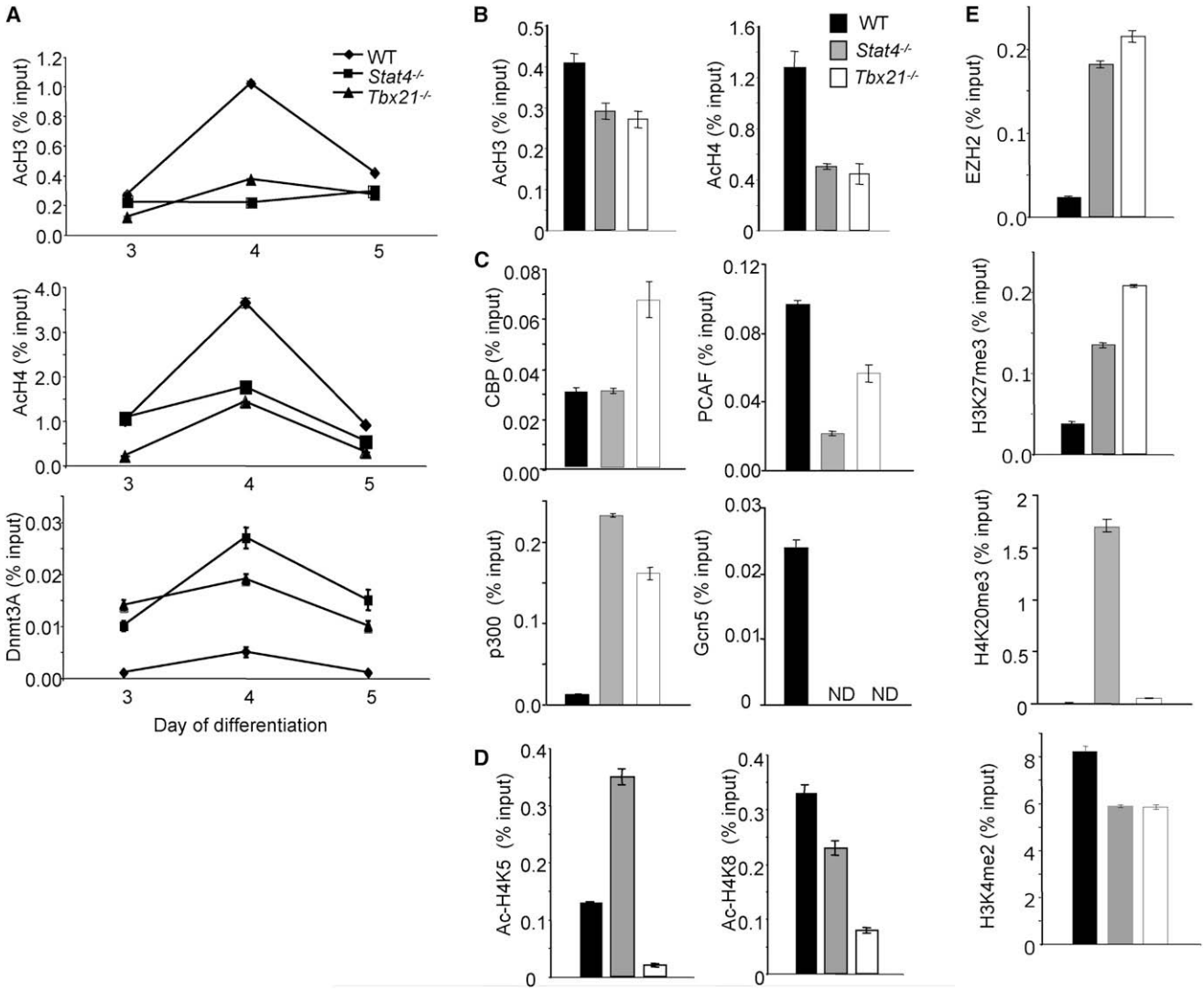


Figure 4. Stat4- and T-bet-Dependent Chromatin Remodeling at the *Hlx1* Locus

(A–E) Wild-type, Stat4-deficient (*Stat4*$^{-/-}$), and T-bet-deficient (*Tbx21*$^{-/-}$) CD4^{+} T cells were cultured under Th1 cell conditions (IL-12 + anti-IL-4) for 5 days, and chromatin was isolated for ChIP assay. ChIP was performed for acetylated-H3, -H4, and DNMT3a on days 3–5 of culture (A) or day 5 only (B); the histone acetyltransferases CBP, p300, PCAF, and Gcn5 on day 5 of culture (C); acetylated H4K5 and K8 on day 5 of culture (D); or EZH2, H3K27me3, H4K20me3, and H3K4me2 on day 5 of culture (E) with quantitative PCR primers for the *Hlx1* promoter. Results are the average ± SD of replicate samples and are representative of three to five experiments for each modification or enzyme with similar patterns. ND, not detected.

a decrease in the association of specific histone acetyltransferases (HATs), we performed chromatin immunoprecipitation (ChIP) assays for HATs at the *Hlx1* promoter. In wild-type cells, the association of HATs at the *Hlx1* promoter did not vary greatly over days 3–5 of differentiation (data not shown). *Hlx1*-associated CBP was not decreased in the absence of Stat4 and was increased in the absence of T-bet compared to that in wild-type cells (Figure 4C). *Hlx1*-associated p300 was increased in both Stat4- and T-bet-deficient cultures compared to wild-type cells. In contrast, association of PCAF and Gcn5 were respectively decreased and undetectable in Stat4- and T-bet-deficient Th1 cell cultures (Figure 4C). The differential effects of Stat4 and T-bet deficiency were also detected in the changes of acetylation at specific histone lysine residues. Whereas overall H4 acetylation was decreased in Stat4-deficient cells, acetylation of H4K5 was, compared to wild-type cells, decreased in T-bet-deficient cells and increased in Stat4-deficient cells (Figure 4D). Acetylation of H4K8 was lower in T-bet-deficient cultures than in Stat4-deficient or wild-type cultures. Thus, although the absence of Stat4 and T-bet results in decreased histone acetylation, each factor has distinct effects on the acetylation of specific histone residues.

We have recently shown that one of the effects of Stat4 activity is to reduce the association of DNMT3a with target loci (Yu et al., 2007). We also observe that DNMT3a has increased association with *Hlx1* in T-bet-deficient cells on days 3–5 of differentiation, though effects were greater in Stat4-deficient cells (Figure 4A). The polycomb group protein EZH2, involved in gene repression through methylaton of H3K27, also had increased association with *Hlx1* in the absence of Stat4, and it had greater association

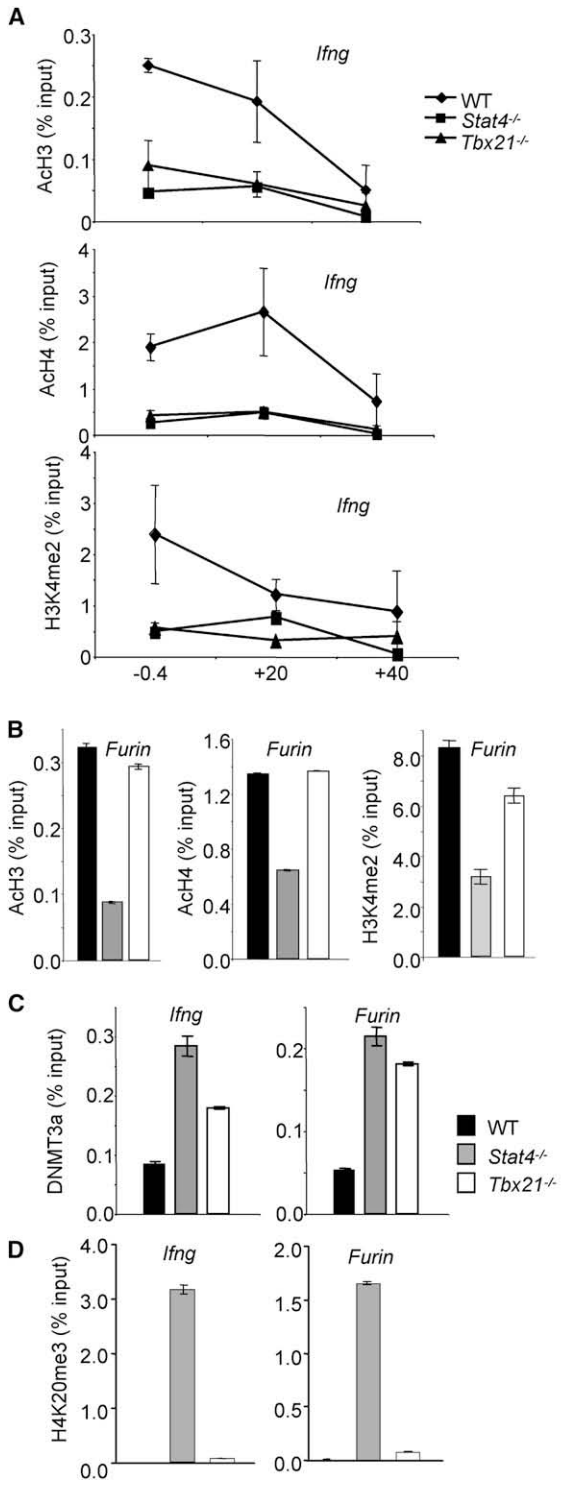


Figure 5. Stat4- and T-bet-Dependent Chromatin Remodeling at Target Loci

(A–F) Wild-type, Stat4-deficient (*Stat4*$^{-/-}$), and T-bet-deficient (*Tbx21*$^{-/-}$) CD4^{+} T cells were cultured under Th1 cell conditions (IL-12 + anti-IL-4) for 5 days, and chromatin was isolated for ChIP assay. ChIP assay was performed for acetylated-H3, -H4, and H3K4me2 at the *Ifng* promoter (−0.4 kb) and at sites +20 kb and +40 kb from the transcriptional start site (A) and at the *Furin* promoter (B). ChIP assay was performed for DNMT3a (C) and H4K20me3 (D) at the *Ifng* and *Furin* promoters. ChIP assay was performed for acetylated-H3, -H4, and H3K4me2 at the *Xcl1* promoter (E) and for acetylated-H4 and H3K4me2 at intron 1 of Cd4 (F). Results are the average ± SD of replicate samples and are representative of two to four experiments for each modification or enzyme with similar patterns.

in *Tbx21*$^{-/-}$ cells, correlating with increased H3K27me3 (Figure 4E). The H4K20me3 modification is also associated with gene repression and was only increased in the absence of Stat4 (Figure 4E). Decreases in H3K4me2 were similar in Stat4- and T-bet-deficient cells, and similar results were observed in cultures initiated from naive CD4^{+} T cells (Figure 4E and Figure S1B). These data demonstrate that Stat4 and T-bet mediate distinct but overlapping changes in chromatin in programming a gene for expression in Th1 cells.

Stat4 and T-bet Remodel Common and Specific Th1 Genes

Because only a subset of the Th1 genes have been analyzed for changes in chromatin structure in the absence of Stat4 or T-bet, we wanted to see whether the transcription-factor-dependent changes we observed at the *Hlx1* locus were also seen at other loci. We examined the *Ifng* gene, which is dependent on both T-bet and Stat4; the *Furin* gene, which was more dependent on Stat4 than T-bet; and the *Xcl1* gene, which was T-bet dependent but Stat4 independent. In agreement with previous reports, acetylated H3 and H4 were decreased in the absence of either Stat4 or T-bet at several sites across the *Ifng* locus (Figure 5A). Moreover, H3K4me2 was highest at the *Ifng* promoter but was decreased in T-bet- and Stat4-deficient cells (Figure 5A). Whereas Stat4 deficiency resulted in decreased Ac-H3, Ac-H4, and H3K4me2 at the *Furin* promoter, these modifications showed only minor changes in the absence of T-bet (Figure 5B). The increased DNMT3a associated with the *Ifng* gene in Stat4- or T-bet-deficient cells was similar to increases observed at the *Hlx1* locus, with slightly greater effect of Stat4 deficiency than T-bet deficiency (Figure 5C). Despite the relative T-bet independence of *Furin* expression, T-bet deficiency also increased DNMT3a presence at the *Furin* locus, suggesting that some effects of T-bet deficiency could be the result of broader changes in factor recruitment (Figure 5C). The increase in H4K20me3 observed at the *Hlx1*

locus in Stat4-deficient cells was also seen at the *Ifng* and *Furin* loci (Figure 5D). In contrast to the Stat4-restricted effects on Furin, we observed T-bet-dependent effects on *Xcl1* (Figure 5E). Histone acetylation and H3K4 dimethylation were decreased in T-bet-deficient cells, but not in cells lacking Stat4 expression. Thus, Stat4 and T-bet not only have distinguishable effects on common target genes, but they further have specific effects on genes that require only one factor for expression in Th1 cells.

To determine whether there are global changes in chromatin modifications and enzyme association, we examined *Cd4* as a common gene that should be independent of Stat4 and T-bet. We did observe a decrease in *Cd4* histone acetylation, although it is important to note that the overall acetylation expressed as percentage of input at this locus is 5-fold higher than that for the Th1 genes examined (Figure 5F). However, H3K4 methylation was unchanged, and DNMT3a association was undetectable at this locus (Figure 5F and data not shown). Moreover, acetylation of the *Il4* locus, a gene that is similarly repressed in these cells, was unchanged among wild-type, *Stat4*$^{-/-}$, and *Tbx21*$^{-/-}$ Th1 cells (data not shown). Thus, there are not global changes in chromatin modifications, although some effects can be observed at genes that do not seem to be direct targets for Stat4 or T-bet.

The Ability of T-bet to Activate the Th1 Genetic Program Requires Stat4

The experiments described thus far indicate that Stat4 and T-bet have separable functions in programming Th1 gene expression. To directly test the ability of T-bet to function in the absence of Stat4, we generated Stat4-T-bet-double-deficient mice. These mice developed normally and had normal thymic and splenic cellularity. Normal T cell development in the thymus and T cell numbers in the periphery were observed in Stat4-T-bet-double-deficient mice (Figure S3). The decreased numbers of natural killer cells and natural killer T cells in the absence of T-bet were also observed in Stat4-T-bet-double-deficient mice but were not affected by additional deficiency in Stat4. Deficiency in protein expression was confirmed by immunoblot of protein extracts from wild-type, Stat4-, T-bet-, and Stat4-T-bet-deficient Th1 cell cultures (Figure 6A). We then examined the expression of Th1 genes in these cultures to determine whether there were any redundant functions of Stat4 and T-bet in the expression of genes that were only partially affected by the absence of Stat4 and/or T-bet (*Lta*, *IL18rap*, and *Runx3*). However, there was not a cumulative effect of deficiency in both Stat4 and T-bet in the expression of any of the Th1 genes examined (Figure 6B).

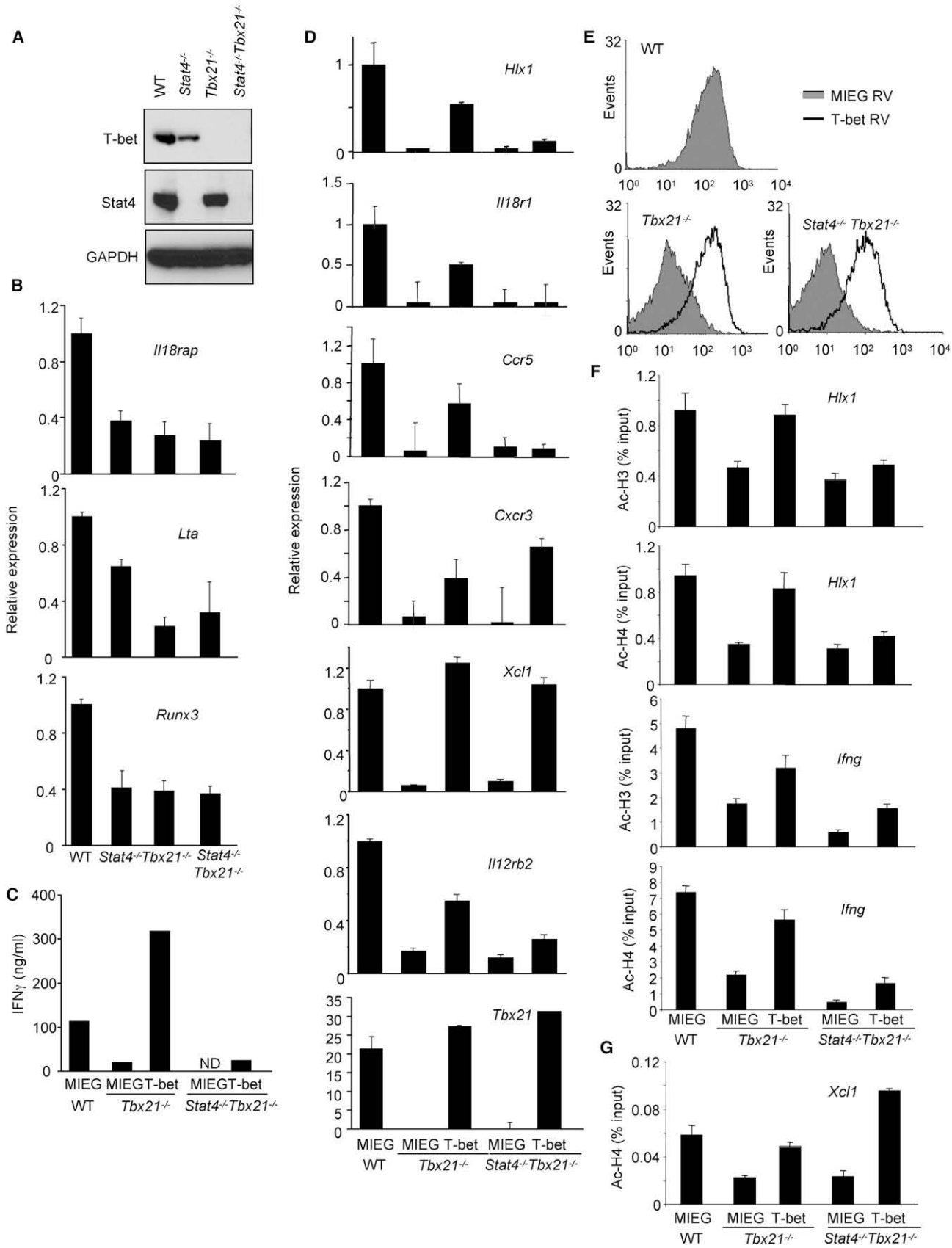
If Stat4 and T-bet perform truly independent functions in the programming of Th1 genes in that each are required for expression, we would expect that T-bet would not be able to activate gene expression in the absence of Stat4. To test this, we transduced T-bet-deficient or Stat4-T-bet-deficient T cells with control or T-bet-expressing retrovirus and compared gene expression to control retrovirus-transduced wild-type cells. Retroviral T-bet expression was fully capable of inducing IFNγ production from T-bet-deficient cells but had only minor effects in transduced Stat4-T-bet-deficient cells (Figure 6C), despite expression of T-bet that was similar to that in wild-type Th1 cells (Figure 6D). Similarly, whereas ectopic T-bet expression could rescue expression of *Hlx1*, *Il18r1*, and *Ccr5* in T-bet-deficient cultures, it had little if any effect in Stat4-T-bet-deficient cultures (Figure 6D). Ectopic T-bet expression minimized the decrease in *Il12rb2* expression observed in *Tbx21*$^{-/-}$ cells, although it had less of an effect in double-deficient cells, correlating with partial Stat4 dependence of this gene (Figure 6D) (Lawless et al., 2000). In contrast, retroviral T-bet expression was able to induce expression of *Xcl1* and *Cxcr3* Stat4-independent genes, in both T-bet-deficient and Stat4-T-bet-deficient cultures (Figures 6D and 6E).

To demonstrate that recovery in gene expression correlates with recovery of histone acetylation mediated by ectopic expression of T-bet, we performed ChIP analysis of Ac-H3 and Ac-H4 in wild-type, T-bet-deficient, or Stat4-T-bet-double-deficient cells transduced with control or T-bet-expressing retrovirus. Histone acetylation was decreased in Stat4-T-bet-double-deficient cells, compared to T-bet-deficient cells at the *Hlx1* and *Ifng* promoters (Figure 6F). Ectopic expression of T-bet increased H3 and H4 acetylation at the *Hlx1* and *Ifng* promoter in *Tbx21*$^{-/-}$ cells, resulting in similar acetylation to that observed in wild-type cells, although T-bet expression had only minor effects on histone acetylation in double-deficient cells (Figure 6F). In contrast, T-bet expression was capable of increasing histone acetylation at the *Xcl1* locus in both single- and double-deficient Th1 cells, similar to the ability of T-bet to promote *Xcl1* expression in *Tbx21*$^{-/-}$ and *Stat4*$^{-/-}$*Tbx21*$^{-/-}$ Th1 cells (Figure 6G). These results demonstrate that there is an intrinsic difference in the ability of T-bet to function at Stat4-dependent and -independent loci and that T-bet requires Stat4 activity to promote chromatin modification and gene expression of the complete Th1 cell phenotype.

DISCUSSION

Transcription factors are critical in regulating the development of effector T cell subsets. Stat4 and T-bet have been extensively characterized for their role in Th1 cell development, but how they functionally interact in the programming of the Th1 genetic signature has not been documented. Many factors have been termed master regulators of developmental pathways, and although these factors are clearly important, it is becoming apparent that they are only part of more complex transcriptional networks. In this report, we have determined that Stat4 and T-bet are not in a linear pathway. Moreover, a decrease of Stat4 expression in T-bet-deficient cells or T-bet expression in Stat4-deficient cells does not alone account for defects in gene expression. First, the identification of genes that depend solely on Stat4 or T-bet suggests that each factor has biological function in the absence of the other factor. Second, the addition of IL-27 does not increase *Ifng* expression in *Stat4*$^{-/-}$ Th1 cell cultures, despite induction of *Tbx21*. Third, binding of Stat4 or T-bet to *Hlx1* is not compromised in the reciprocal gene-deficient cells. We further demonstrate that although T-bet is able to induce chromatin modifications and mRNA of T-bet-dependent, Stat4-independent genes, T-bet is unable to activate Stat4-dependent genes in the absence of Stat4. This demonstrates that both transcription factors are needed for the development of the complete Th1 cell phenotype.

These data raise the question of the temporal requirements for Stat4 and T-bet to function as chromatin-remodeling factors or


A
WT
Stat4-/-
Tbx21-/-
Stat4-/-Tbx21-/-
T-bet
Stat4
GAPDH
B
Il18rap
Lta
Runx3
Relative expression
WT
Stat4-/-Tbx21-/-
Stat4-/-Tbx21-/-
C
IFNγ (ng/ml)
ND
MIEG
MIEG T-bet
WT
Tbx21-/-
Stat4-/-Tbx21-/-
D
Hlx1
Il18r1
Ccr5
Cxcr3
Xcl1
Il12rb2
Tbx21
Relative expression
E
WT
MIEG RV
T-bet RV
Events
Tbx21-/-
Stat4-/- Tbx21-/-
F
Ac-H3 (% input)
Ac-H4 (% input)
Hlx1
Ifng
G
Xcl1

as factors that interact with the transcriptional machinery in the appropriate chromatin environment. After Stat4 binds to a gene, it mediates histone hyperacetylation and alters other histone modifications and chromatin-associated enzymes (O'Sullivan et al., 2004; Yu et al., 2007; Yu et al., 2008) (this report). Through these functions, Stat4 also results in increased transcription of target loci (O'Sullivan et al., 2004; Yu et al., 2007). During the differentiation period, the addition of IL-12 on the third day of culture results in an increase in mRNA and acetylated histone of *Il18r1*, *Hlx1*, and probably other genes as well (Yu et al., 2007). However, because Stat4 is only transiently activated, it is unlikely that Stat4 needs to remain bound to target loci to maintain gene expression. The role of T-bet is less clear, with reports showing T-bet dependence and T-bet independence of epigenetic modification of the *Ifng* locus (Avni et al., 2002; Mullen et al., 2002; Usui et al., 2006). Our studies demonstrate the ability of T-bet to induce histone acetylation in the context of Stat4. A recent report using an inducible form of T-bet suggested that stable T-bet activity, but not transient T-bet activity, was required for maintaining gene expression (Matsuda et al., 2007). Thus, T-bet may induce remodeling, but it is also a direct activator of transcription. Indeed, we observed that in the presence of inhibitors that block repressive chromatin and DNA modifications, *Hlx1* gene expression is increased in *Stat4*$^{-/-}$ Th1 cell cultures, but not *Tbx21*$^{-/-}$ Th1 cell cultures, suggesting that transcription depends upon the presence of T-bet.

The degree to which Stat4 and T-bet regulate each other's expression has been examined in a number of reports. One particularly contentious point is whether T-bet expression is decreased in the absence of Stat4 (Mullen et al., 2001; White et al., 2001; Afkarian et al., 2002; Hoey et al., 2003), and there are several explanations for discrepancies among these reports. First, although IFNγ and Stat1 efficiently induce *Tbx21* expression, recent reports do support a lesser role for Stat4 in activating *Tbx21* (Usui et al., 2006; Yang et al., 2007). Second, the time during differentiation and the activation state of the cells have an impact on the difference in expression. Furthermore, differences in culture systems, such as the use of purified T cells versus the use of TCR transgenics in which antigen-presenting cells (APCs) are present, may affect results. APCs might provide cytokines, including IL-27, or other costimulatory signals that affect *Tbx21* expression independent of the IL-12-induced Stat4 signal. Similarly, culture conditions and the cytokine environment might affect Stat4 expression in the absence of T-bet (Usui et al., 2006). Importantly, even in conditions in which decreases in Stat4 or T-bet expression are observed, changes are not dramatic. Moreover, as we have shown, the modest decreases in the expression of either factor do not negatively affect the ability of each factor to bind at least one target gene, *Hlx1*, in the absence of the reciprocal factor or induce the subsets of Th1 genes and chromatin modifications that are differentially dependent on either factor.

Changes in chromatin that mediate gene programming are necessarily complex. Although routine examinations of acetylated histones H3 and H4 define the protein's overall acetylation, which largely correlates with transcription at the locus, these analyses lack the resolution of examining specific chromatin modifications and the recruitment of chromatin-modifying complexes to specific loci. Whereas we observed that overall histone acetylation was decreased in the absence of Stat4 or T-bet and that retroviral expression of T-bet induced histone acetylation when endogenous Stat4 was present, specific H4 residues actually had increased acetylation in the absence of Stat4. Moreover, the increase in *Hlx1*-associated p300 in Stat4- and T-bet-deficient cells and the increase in *Hlx1*-associated CBP in T-bet-deficient cells highlight that the recruitment of these enzymes is not dependent on either factor at this locus and that associated CBP or p300 do not always correlate with total acetylation or gene expression. In contrast, both Stat4 and T-bet contributed to the recruitment of PCAF and Gcn5, components of large histone-remodeling complexes including STAGA, TFTC, and PCAF (Lee and Workman, 2007; Nagy and Tora, 2007), and association of these factors correlated with the overall acetylation of H3, H4, and specifically H4K8. This is similar to the ability of Gcn5-PCAF, but not CBP-p300, to acetylate H4K8 in the context of the IFNβ gene (Agalioti et al., 2002). Thus, the recruitment of specific HAT complexes is required for *Hlx1* gene expression.

In addition to regulating histone acetylation, Stat4 and T-bet also regulate the recruitment of other enzymes that generate chromatin modifications associated with either gene activation or gene repression. Stat4, but not T-bet, mediates the recruitment of Brg1-containing SWI/SNF complex to the *Ifng* locus (Zhang and Boothby, 2006). Moreover, T-bet recruits H3K4 methylases, which include Set7/9, to the *Ifng* and *Cxcr3* loci (Shnyreva et al., 2004; Lewis et al., 2007). We observe a similar requirement for T-bet in mediating this modification at *Hlx1, Ifng*, and *Xcl1* genes, and we also show that Stat4 promotes H3K4 methylation at target loci. T-bet limits the association of repressive complex proteins such as mSin3a, whereas T-bet and Stat4 prevent the recruitment of DNA methyltransferases and DNA methylation of target loci, although DNMT3a association is more affected by Stat4 deficiency (Mullen et al., 2002; Tong et al., 2005; Yu et al., 2007). In the absence of either Stat4 or T-bet, there are increases in EZH2 associated with the locus and increased H3K27me3, whereas increases in H4K20me3 were specifically found in the absence of Stat4. Changes to target loci are the result of transcription factors changing the equilibrium of positively and negatively acting factors associated with local chromatin. Moreover, chromatin alterations that affect

Figure 6. Stat4 Requirement in T-bet Function

(A) Wild-type, Stat4-deficient (*Stat4*$^{-/-}$), T-bet-deficient (*Tbx21*$^{-/-}$), and Stat4-T-bet-double-deficient (*Stat4*$^{-/-}$*Tbx21*$^{-/-}$) CD4$^+$ T cells were cultured under Th1 cell conditions (IL-12 + anti-IL-4) for 5 days, and total cell extracts were immunoblotted for T-bet, Stat4, and GAPDH as a control.

(B) Cells cultured as in (A) were assessed for the expression of Th1 genes before (*Il18rap* and *Runx3*) or after (*Lta*) restimulation with anti-CD3.

(C–G) Wild-type, T-bet-deficient (*Tbx21*$^{-/-}$), and Stat4-T-bet-double-deficient (*Stat4*$^{-/-}$*Tbx21*$^{-/-}$) CD4$^+$ T cells were cultured under Th1 cell conditions. On day 2 of the culture period, cells were transduced with a bicistronic retrovirus expressing EGFP only (MIEG) or T-bet and EGFP (T-bet). At the end of the culture, cells were sorted for EGFP expression and stimulated for 18 hr with anti-CD3. Supernatants were analyzed for IFNγ with ELISA (C). RNA was isolated from each population for determining the expression of the indicated genes with qPCR (D). Surface expression of CXCR3 was determined with flow cytometry (E). ChIP assay was performed for acetylated-H3 or -H4 at the *Hlx1* and *Ifng* promoters (F) or acetylated-H4 at the *Xcl1* promoter (G). Results are the average ± SD of replicate samples and are representative of two to three experiments with similar results.

gene transcription can occur at a distance, as evidenced by recent extensive analyses of the *Ifng* locus (Chang and Aune, 2007; Schoenborn et al., 2007), and may depend on transient changes to a target locus as well. A further understanding of the hierarchy of chromatin-modifier association to target loci, in the presence or absence of Stat4 and T-bet, should provide insight into how genes are programmed during T cell differentiation.

These data further suggest that Th1 gene expression and function could be heterogeneous depending on the cytokine environment to which developing cells are exposed. In a milieu with high IFNγ, and therefore high T-bet but low IL-12, T cells should still be programmed with expression of the Stat4-independent genes. Cells derived in this environment would have low expression of IFNγ but normal expression of Egr2 and Egr3, which promote FasL expression (Rengarajan et al., 2000). Moreover, they would express CXCR3 and XCL1, allowing them to be recruited, and to recruit to, sites of inflammation. It is not yet clear whether there is a gradient or a threshold for gene programming by these factors. If a threshold exists, we would expect distinct cell states, cells with sufficient T-bet activation to program Th1 genes but not sufficient Stat4 activation, cells with sufficient Stat4 activation but with reduced T-bet activation, and cells with sufficient activation of both factors. However, a model in which there could be a gradient of effects of either factor would predict even greater heterogeneity in the Th1 cell response, allowing increased programmatic flexibility in responding to specific pathogens.

T-bet has been termed a master regulator of the Th1 cell phenotype. In this report, we demonstrate that T-bet does not act alone, that Stat4 is also required to modify chromatin and establish the full Th1 cell phenotype. The need for Stat4 in this process may be direct, by its binding to genes and altering the chromatin environment, and also indirect, by, for example, its inducing the expression of Hlx and Runx3, which have been shown to functionally cooperate with T-bet in promoting *Ifng* expression (Mullen et al., 2002; Djuretic et al., 2007). In addition to their complementary roles in Th1 cell differentiation, Stat4 and T-bet regulate the development of other Th subsets, including Th17 (Mathur et al., 2006; Mathur et al., 2007; Furuta et al., 2008), as well as playing important roles in innate immune cells. It will be important to define the precise mechanisms of transcriptional regulation that involve Stat4 and T-bet in these other cell types to determine whether they are similar or whether context-dependent functions result in appropriate transcriptional regulation.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 *Stat4*$^{-/-}$ and *Tbx21*$^{-/-}$ (Taconic Farms, Germantown, NY, USA) mice have been previously described (Kaplan et al., 1996; Szabo et al., 2002). C57BL/6 mice were purchased from Harlan Bioproducts (Indianapolis, IN, USA). *Stat4*$^{-/-}$*Tbx21*$^{-/-}$ mice were generated by intercrossing single-deficient mice. Mice were maintained under specific pathogen-free conditions. All experiments were performed with the approval of the Indiana University Institutional Animal Care and Use Committee.

In Vitro T Cell Differentiation and Analysis of Gene Expression

CD4 cells were isolated from spleen and lymph nodes of mice with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). For Th cell differentiation, CD4 cells (1×10^6 cells/ml) were cultured with plate-bound anti-CD3 (4 μg/ml) and 0.5 μg/ml soluble anti-CD28 under Th1 cell (2 ng/ml IL-12 and 10 μg/ml anti-IL-4) or Th2 (10 ng/ml IL-4 and 10 μg/ml anti-IFNγ) skewing conditions and expanded after 3 days. In some experiments, 100 ng/ml IFNγ or 100 ng/ml IL-27 were added as described. After 5 days of culture, cells were harvested for gene expression or restimulated with anti-CD3 for ELISA. Quantitative RT-PCR was performed as described (Mathur et al., 2006). Message RNA was analyzed with TaqMan PCR reagents specific for each of the indicated genes (Applied Biosystems, Foster City, CA, USA). Cycle numbers of duplicate samples were normalized to expression of β2-microglobulin. Expression of some genes was examined after activation with anti-CD3 for 6 hours (*Ifng*, *Xcl1*, *Egr2*, *Egr3*, *Furin*, and *Lta*) when mRNA in resting cells was very low or undetectable. Immunoblot and ELISA were performed with standard methods (Mathur et al., 2006; Yu et al., 2007).

Chromatin Immunoprecipitation

ChIP assay was performed as previously described (Yu et al., 2007) with minor modification. In brief, crosslinking of protein-chromatin complexes was achieved by adding formaldehyde into cell cultures to a final concentration of 1%. Cells were washed in phosphate-buffered saline, resuspended in cell-lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% SDS), and incubated for 10 min on ice. An ultrasonic processor (Vibra-Cell, Sonics & Materials, Newtown, CT, USA) was used to shear genomic DNA (150–300 bp fragments), with ten 10 s 70 W bursts. Cell extracts were diluted in ChIP buffer, precleared with salmon sperm DNA, bovine serum albumin, and protein A agarose bead slurry (50%) at 4°C for 1 hr. The supernatant was incubated in the presence or absence of 5 μg antibody (anti-Stat4, anti-T-bet, anti-Dnmt3a, anti-HAT, anti-p300, anti-CBP, and anti-PCAF [Santa Cruz Biotechnology, Santa Cruz, CA, USA]; anti-acetylated H3, anti-acetylated H4, and anti-H4K20me3 (Millipore, Billerica, MA, USA); and anti-H4K5, anti-H4K8, and anti-Ezh2 [Abcam, Cambridge, MA, USA]) at 4°C overnight. The immunocomplex was precipitated with protein A agarose beads at 4°C for 2 hr, followed by centrifugation. The supernatant from the control precipitation was used as input material. The beads were washed consecutively with low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and twice in TE buffer. Bound DNA was eluted from the beads twice with elution buffer (0.1 M $NaHCO_3$, 1% SDS) by rotating at room temperature for 15 min. The supernatant was collected, supplemented with 2 mM EDTA, 20 mM Tris-Cl, and 10 mg/ml Proteinase K, and incubated at 37°C. DNA crosslinks were reversed by incubating precipitates at 65°C for 16 hr. DNA was purified by phenol-chloroform extraction and ethanol precipitation and was resuspended in H_2O. Real-time quantification of ChIP assay was done as previously described, with TaqMan primer sequences previously reported (Yu et al., 2007) or primers for SYBR Green as listed in Table S2. For quantification of chromatin immunoprecipitates, a standard curve was generated from serial dilutions of a known amount of sonicated Th1 cell DNA. For calculation of ChIP results as a percentage of input, the amount of the immunoprecipitated DNA from the isotype control antibody was subtracted from the amount of the immunoprecipitated DNA from the specific antibody ChIP, followed by normalizing against the amount of the input DNA with quantitative PCR. Data are shown as percentage input from a representative of two to four experiments.

Retroviral Transduction

Purified $CD4^+$ T cells were cultured under Th1 cell conditions, and on day 2, cells were transduced with a bicistronic retrovirus expressing EGFP only (MIEG) or T-bet and EGFP (T-bet) in the presence of 20 units/ml of IL-2 as previously described (Chang et al., 2005; Mathur et al., 2006). After transduction, cells were rested at 37°C for 2 hr and cultured under Th1 cell conditions for another 3 days prior to flow cytometry or cell sorting for ELISA and real-time PCR application. Flow-cytometric analysis was performed according to standard methods with a PE-labeled anti-CXCR3 (R&D Systems, Minneapolis, MN, USA) (Mathur et al., 2007). For analysis of histone acetylation, cells were fixed directly after sorting, and ChIP analysis was performed as described above with the addition of normalizing results for control of analysis of Cd4.

SUPPLEMENTAL DATA

Supplemental Data include two tables and three figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00461-5.

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